This draft, January 2001, prepared by EH-6, has not been approved and is subject to modification. Project No. SDMP-0029

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METRIC

DOE-SPEC-XXXX-YEAR
PROPOSED

DOE SPECIFICATION

BERYLLIUM LYMPHOCYTE PROLIFERATION TESTING (BeLPT)

U.S. Department of Energy
Washington, D.C. 20585

AREA SDMP

FOREWORD

1. Use of this purchase specification is not mandatory. User should review the document and determine if it meets the user's purpose.

2. Comments (recommendations, additions, and deletions) and any pertinent data that may be of use in improving this document should be addressed to: DOE Beryllium Lymphocyte Proliferation Testing Writing Group, c/o Paul Wambach, EH-6 /GTN, U.S. Department of Energy, 19901 Germantown Road, Germantown, MD 20874-1290.

3. This document was developed from a draft protocol “Testing for Sensitivity to Beryllium and Investigating Chronic Beryllium Disease” that was distributed in June 2000 by Dr. Frederick Miller, Chairman of the Committee to Accredit Beryllium Sensitivity Testing (CABST) Committee.

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PARAGRAPH PAGE

1. SCOPE. This specification is for beryllium lymphocyte proliferation tests (BeLPT) used for detecting an individual's sensitivity to beryllium and for clinical evaluation and diagnosis of patients for chronic beryllium disease. This specification should be used in all contracts with laboratories for the purchase of BeLPT Services.

2. BACKGROUND. Beryllium is a lightweight metal that can cause a chronic granulomatous disease called chronic beryllium disease (CBD). Development of this disease process is a function of exposure, an individual's ability to mount a beryllium-specific, cell-mediated immune response to beryllium (called sensitivity), their ability to develop granulomatous responses and possibly other factors.

   Testing for an individual's sensitivity to beryllium using an in-vitro assay is currently used: as a screening assay; as part of the diagnostic criteria for chronic beryllium disease; and for surveillance in identifying unhealthy working conditions. The following protocols have been developed to optimize and standardize beryllium sensitivity testing.

   These procedures have been shown to require some degree of experience. Individuals requesting studies dealing with beryllium sensitization or CBD status should employ facilities with an established and continuing record of satisfactory performance.

3. PRINCIPLE

   3.1. When a lymphocyte T cell antigen receptor recognizes a specific antigenic substance bound to an HLA Class I or II molecule, the T cell responds in a variety of ways (for example secreting inflammatory cytokines and/or undergoing cell division). Each T lymphocyte expresses only a single T cell antigen receptor. When sufficient numbers of specific T cells are present (usually greater than l/10,000), the response of specific T cells can be detected clinically by either in-vitro or in-vivo testing. In-vivo, this response is usually measured as a delayed hypersensitivity skin test. In-vitro, the T cell response is normally measured by recording the proliferation response of the cells. When an individual has a clinically measurable response to a specific antigen, that individual is said to be sensitive or hypersensitive to that antigen.

   3.2. Beryllium can be a component of antigens and is associated with a granulomatous hypersensitivity disorder in a small number (ca 5%) of individuals in an exposed population. Large numbers of CD4+ T-lymphocytes accumulate in the lung in chronic beryllium disease (Rossman et al, 1988 and Saltini et al, 1989). The reactivity of these lymphocytes to beryllium provides a specific and sensitive laboratory test for differentiating chronic beryllium disease from sarcoidosis (Rossman et al, 1988). Only CD4+ T-lymphocytes appear to react to beryllium (Saltini et al, 1989). In chronic beryllium disease, the beryllium reactivity of lymphocytes obtained by bronchoalveolar lavage is generally greater than the reactivity of lymphocytes obtained from the peripheral blood. By measuring the reactivity of lymphocytes obtained from the peripheral blood, beryllium sensitivity can also be detected in workers without disease (Kreiss et al, 1993). Some of the workers without disease

that have been identified by positive blood proliferation responses to beryllium have gone on to develop disease.

4. APPLICATION. Beryllium sensitivity testing is used as a screening tool for possible chronic beryllium disease, as a surveillance tool in indicating hazardous working conditions, and as part of the diagnostic criteria for the disease. The blood lymphocyte proliferation test for beryllium sensitization (BeLPT) is a screening test with a sensitivity and specificity that is not clearly defined at this time. This is attributable to the fact that the populations of normal people have not had lung biopsy and bronchoalveolar lavage to include or exclude chronic beryllium disease. The positive predictive value can be calculated and is estimated to be approximately 44 to 50 percent. The bronchoalveolar lavage lymphocyte proliferation test for beryllium sensitization (BAL-LPT) is the preferred test of beryllium sensitivity as part of the diagnostic criteria for chronic beryllium disease.

5. TRITIATED THYMIDINE UPTAKE PROCEDURE FOR DETERMINING BERYLLIUM SENSITIZATION

5.1. REAGENTS AND EQUIPMENT

5.1.1. RPMI-1640 medium supplemented with 25mM HEPES buffer and 200 mM L-glutamine

5.1.2. Human serum, Type AB, heat inactivated to eliminate complement activity. Each lot MUST BE TESTED for its ability to support lymphocyte proliferation (i.e., it should not exhibit excessive levels of toxicity or cell killing in the presence of beryllium). It is aliquoted and stored at -20°C until use. It should not be refrozen.

5.1.3. Penicillin-streptomycin, 100 U and 50 μgm/ml respectively or 50 μg/ml gentamicin sulfate

5.1.4. Phosphate-buffered balanced salt solution, calcium, and magnesium-free (i.e., Hank's, Dulbecco's, etc.)

5.1.5. Tritiated Thymidine (specific activity 2-10 Ci/mM)

5.1.6. Beryllium sulfate, 0.2 M solution (Brush Wellman, Inc.)

5.1.7. PHA (Phytohemagglutinim) and/or Con A (concanavalin-A) and/or Candida albicans allergenic extract or recall antigen suitable for population such as tetanus. Should be prepared in a concentration known to stimulate lymphocyte proliferation optimally (i.e.,~ 30 μg/ml in culture and 10 μg/ml in culture for PHA and ConA, respectively).

5.1.8. Density gradient solution for blood mononuclear lymphocyte separation (i.e., Ficoll-Hypaque, Histopaque, etc.). Once opened, store at 4°C and warm to room temperature before use. (For Blood LPT only).

5.2. PREPARATION OF REAGENTS

5.2.1. Complete growth medium (should be prepared using sterile technique) to a final concentration of 10% human AB serum, 1% L-glutamine, and 1% penicillin-streptomycin in RPMI-1640 (e.g., to 1 x 500 ml bottle RPMI-1640 add 55 ml human AB serum, 5.5 ml L-glutamine [200 mM] and 5.5 ml penicillin-streptomycin [100 x]). Prepare the quantity that will be used in a week. Store at 4°C.

5.2.2. Tritiated Thymidine (thymidine, [methyl-^{3}H])). Note: Since tritiated thymidine is a radioactive material, appropriate safety precautions must be applied to prevent spills, to properly label and dispose of contaminated pipettes, vials, microtiter plates and other items in contact with this substance as well as all liquid wastes. Laboratory-specific requirements for technician training and waste disposal should be developed in consultation with the appropriate radiation safety officer. Prepare a new lot number of tritiated thymidine in the following manner: dilute 1 mCi/ml of tritiated thymidine with the medium prepared in step 6.2.1 (e.g., 1 ml tritiated thymidine + 19 ml Medium).

5.2.3. Beryllium Sulfate ($BeSO_4$), store at room temperature. Same procedure for other salts of beryllium if used. Prepare ($BeSO_4$) dilutions fresh for each assay, using the 0.2 Molar (M) stock solution. Sterilize the solutions by filtration.

5.2.3.1. Make a 1:10 dilution of ($BeSO_4$) solution with phosphate buffered saline solution, Ca++ and Mg++ free.

5.2.3.2. Continue making serial dilutions with Complete Medium to create three dilutions of 2 µM $BeSO_4$, 2 0µM $BeSO_4$, and 200 µM $BeSO_4$. When added to wells, this will give a range of concentrations of beryllium from 1, 10, and 100 µM $BeSO_4$.

5.3.QUALITY CONTROL

5.3.1. New lots of AB serum are tested for ability to support a response to beryllium. Specifically, the serum should produce stimulation indexes of approximately 1 in beryllium-challenged wells for non-sensitized subjects. Also, it should demonstrate low toxicity or cell-killing [i.e., not more than one standardized Ln(SI) (See B.6.) should be less than –3.0] in the presence of beryllium. The serum should NOT stimulate excessive lymphocyte proliferation in control wells.

5.3.2. The beta counter (gas ionization or scintillation) should be recalibrated at regular intervals according to the instrument manufacturer's specifications to ensure optimal performance. Documentation of the calibration should be kept in a readily accessible format.

5.3.3. Each plate contains blank wells. Mean counts per minute (cpm) for the blank wells should not exceed acceptable background for the counting instrument used. Documentation of background counts should be maintained.

5.3.4. After intensive training, each new technician should set up approximately 10 tests in duplicate with other experienced technicians. This can be done using blood submitted, since about 25% of the samples will have enough cells. When the duplicate tests are completed, the following parameters should be compared: counts in blank wells, coefficient of variation in the control wells, calculated stimulation indexes in the beryllium-stimulated wells and in the mitogen-stimulated wells, and overall interpretation of the test. The comparison should be based on the statistics described in Appendix B.

5.3.5. The laboratory should demonstrate knowledge of and adherence to accepted practices for dealing with chemical, biological, and radiological hazards. Documentation should be maintained on laboratory personnel training in the areas of: radiation hazards, blood-borne pathogens, and chemical hazards.

5.3.6. The laboratory should maintain a log of samples received containing, at a minimum: name and/or identification number, date received, time in transit, and unusual circumstances (e.g., more than 24 hours old, hemolyzed, tubes extremely hot or cold, etc.).

5.3.7. It shall be the responsibility of the Laboratory Director or designee to assure that the laboratory meets an external standard of quality assurance and quality control and that the laboratory is in compliance with appropriate State and Federal regulations. The College of American Pathologists (CAP) accreditation is highly recommended.

6. TRITIATED THYMIDINE BeLPT: BLOOD SPECIMEN.

6.1. SAMPLE COLLECTION PROCEDURE:

6.1.1. For the blood BeLPT, 30 ml heparinized blood is sent at room temperature to arrive at the testing site within 24 to 30 hours of being drawn. Appropriate insulating material should be used to maintain satisfactory temperature control and to avoid extreme temperature fluctuation of the cells and maximize cell viability during shipping. Use three sterile green-top sodium heparinized

vacutainers, 10 ml size. Cell separation must be achieved within 36 to 48 hours at the very outside.

6.1.2. The tubes should be labeled with patient's name, date and time blood is drawn, identification number, name of person obtaining specimen at a minimum.

6.1.3. Neither the blood specimen nor the packing materials should ever be refrigerated. Shipping containers should be labeled: HUMAN BLOOD DELIVER IMMEDIATELY. DO NOT FREEZE, PERISHABLE. Biosafety packing should be utilized.

6.2. SAMPLE PREPARATION

6.2.1. Separate the lymphocytes by centrifugation on a density gradient medium using a method that will maximize the yield of mononuclear cells. Aspirate the cell interface with a sterile pipette. Wash the cells three times in approximately 10 ml of buffered saline. Perform a cell count to determine the amount of complete medium to add so that the final cell concentration is 2.5 X 10^5 cells/0.1 ml. Each well will receive 0.1 ml of the cell suspension and 0.1 ml of mitogen, antigen, beryllium concentration or complete medium to bring the well volume to 0.2 ml per well.

6.2.2. The recommended plate arrangement requires approximately 6ml of cell suspension (concentration of 2.5 x 10^6/ml) distributed into 4-12 wells each of:

6.2.2.1. At least 4 treated wells for each of the 3 concentrations of $BeSO_4$, to be harvested on two separate days, from day 4 to day 7.

6.2.2.2. 8 to 12 Control wells, 0.1 ml/well (cells in complete medium only, harvest correspond to harvest days chosen in 6.1.2.1.1). If two sera are used and the harvest is on one day, then control wells will be seeded using each serum.

6.2.2.3. 4 Mitogen/antigen #1wells (harvest on the optimal day)

6.2.2.4. 4 Mitogen/antigen #2 wells (harvest on optimal the day)

6.2.3. Incubation for the number of days selected shall be carried out at 37°C in an atmosphere of 5-10% carbon dioxide-air.

6.2.4. At 6 to 18 hours prior to each harvest, add 1 μCi tritiated thymidine to each well. Return plate to incubator.

6.2.5. Harvest cultures using a 96 well harvester and filter mats. Cells will be collected in a standard glass filter fitted harvester or its equivalent and counts

measured in a beta emission scintillation (using a suitable cocktail) or gas ionization counter expressing the data in cpm. Counting time should be at least 1 minute per tube if a beta emission scintillation counter is used and 4 minutes per filter if a gas ionization counter is used.

7. TRITIATED THYMIDINE BeLPT: BRONCHOALVEOLAR LAVAGE (BAL) SPECIMEN

7.1. SAMPLE COLLECTION.

7.1.1. For the BAL-LPT, the specimen is a saline lavage obtained during bronchoscopy. It is recommended that samples be taken from the wedged position in lingula or right middle lobe. The lavage is preformed before the biopsy to avoid contamination with blood. A minimum of 200 ml normal saline is instilled in 25-60 ml aliquots. Gentle aspiration occurs after each aliquot is instilled. Fluid recovery usually varies from 50- 70%. The specimen must be transported to the laboratory immediately. Temperature should be kept between 15 to 25°C using insulation if necessary.

Specimens that cannot be set up for proliferation testing that day must be suspended in RPMI-1640 with antibiotics and processed within 36 hours (no more that one night should pass before the cells are placed into culture for proliferation assays.) The test requires a minimum of 2.5×10^6 cells and 20×10^6 are recommended.

7.1.2. BAL preparations from normal individuals usually consist of over 90% alveolar macrophages. However, in beryllium disease higher percentages of lymphocytes are typically observed. BAL fluid is centrifuged, the cells re-suspended in culture medium, counted and exposed to beryllium for 3 and 5 days. The presence of immuno-competent T lymphocytes in the preparation is usually ascertained by testing with Candida albicans, a common recall antigen although comparable universal antigens/mitogens may be employed to the same end. Tritiated thymidine is included for the last 6-18 hrs. of culture and the assay is terminated by harvesting cells onto filter paper and determining the radioactive content in a scintillation or gas ionization counter.

7.1.3. SAMPLE FORM

7.1.3.1. PATIENT NAME: BIRTH DATE:

7.1.3.2. DATE AND TIME OF TEST: SW:

7.1.3.3. SPECIMEN #

7.1.3.4. LAVAGE VOLUME- ml IN:

7.1.3.5. HOSPITAL#

7.1.3.6. ml OUT: %Return:

7.1.3.7. CELL YIELD: Total number of cells recovered/ml lavage fluid out

7.1.3.8. Lymphocytes _____%; Alveolar macrophages _____%
Eosinophils _____ % Neutrophils _____%;
Basophils _____%; RBC/WBC _____

7.1.3.9. BROWNISH INTRACELLULAR MATERIAL?: (YES/NO) (indicates smoker)

7.1.3.10. NUCLEAR DEBRIS? (YES/NO) (indicates problems with lavage procedure)

7.2. ANALYTICAL PROCEDURE. After the lavage sample has been received in the LPT laboratory.

7.2.1. Note volume of lavage on summary sheet. Give a brief description of the fluid (cloudy, bloody, etc.).

7.2.2. Mix fluid and remove 3 - 5 ml of lavage for CBC and differential.

7.2.3. Transfer lavage to 50 ml centrifuge tube(s), and spin for 5 minutes at 400 x g. Repeat twice, washing the cells three times, to be certain all surfactant proteins are removed. A calcium and magnesium-free balanced salt solution such as Hanks or Dulbeccos should be used for the washings.

7.2.4. After washing, re-suspend the cell pellet in the complete medium to a concentration to 1.0×10^6 mononuclear cells/ml. Dispense 0.1 ml of cell suspension into wells of the plate. This provides 1×10^5 cells/well. Higher cell concentrations may produce lower counts (diminished stimulation) in the BAL-LPT.

Note: It may be difficult to obtain sufficient cells to achieve the required final cell concentration. Accordingly, the assay should be performed on the original lavage cell preparation and a Ficoll-Hypaque separation is done only if there are sufficient cells. The cell recovery in the gradient is considerably less than 100%. Laboratory experience in cell recovery should be the guide. In patients with end-stage lung disease there are often increased numbers of neutrophils which can be removed by Ficoll-Hypaque gradient centrifugation (if PMN>20%).

7.2.5. The recommended plate arrangement requires 6×10^6 mononuclear cells in 6.0 ml medium. When sufficient cells are available, the LPT on the lavage

sample should be set up and harvested as described for the peripheral blood LPT (see paragraphs 7.2.3, 7.2.4, and 7.2.5).

7.2.6. lf cell yield is low, the minimum acceptable assay is:

4 wells of each of the following: (Be concentration 1, be concentration 2, be concentration 3, and control—16 total wells)

4 wells of PHA with at least 2 x 10^6 cells are required

Only an abnormal response would be acceptable in this circumstance.

8. CALCULATING BeLPT METRICS

The BeLPT is based on alpha particle counts from the tritiated thymidine that determine the degree of lymphocyte proliferation. The data is analyzed by comparing counts from $BeSO_4$ exposed wells and mitogen exposed wells to the counts from unexposed control wells. The laboratory must calculate the metrics it uses to determine if a test is acceptable, and whether the result is normal, abnormal, or borderline. Electronic copies of count data and all calculations must be provided to the purchaser upon request. Appendix B describes the least absolute value (LAV) method of performing the required calculations. Steps in the calculations are described in detail with a numerical example. It also describes assumptions and possible alternative methods of analysis. A laboratory that chooses an alternative method must include a description of the method. The steps in the calculation based on the LAV method are summarized here.

8.1. For each treatment group, calculate a metric called a "stimulation index" (SI) that is a ratio of the response in treated wells to the response in unexposed control wells (see Appendix B.3).

8.2. For each treatment group, calculate a metric called a standardized natural log SI [standardized Ln(SI)] that uses the amount of well-to-well variance in the test to estimate the standard error of the Ln(SI). The standardized Ln(SI) is obtained by dividing the Ln(SI) by its standard error (see Appendix B.4-B.6). This standardized statistic indicates the extent to which the Ln(SI) differs from the reference value of zero. Large (i.e. greater than 2.5) positive values indicate a response to beryllium.

8.3. Establish a reference data set for each serum, and determine the Ln of the maximum SI for each BeLPT. Calculate the median (M) and standard deviation (SD) of the Ln(maximum SI) for the reference data set (see Appendix B.10).

8.4. Calculate a metric called the "standardized maximum Ln(SI)". First, find the treatment group with the largest Ln(SI), i.e., the maximum Ln(SI). Next, subtract the value of M for the reference data set and divide by the standard deviation, i.e. [maximum LN(SI)-M]/SD. This metric compares the strongest response for each BeLPT with the strongest responses from normal individuals in the reference data set.

9. RESULTS. The results of BeLPTs shall be reported as unacceptable, abnormal, normal, or borderline. If a test is unacceptable, it will be repeated. If a test is abnormal or borderline, then two duplicate repeat BeLPTs will be requested. Usually these tests will be performed in two different laboratories or in one laboratory utilizing two different sera. If at least two of the three BeLPTs are abnormal the patient is deemed beryllium sensitized. Since the criteria for a single abnormal BeLPT is based on approximate false positive probability of 0.001, the chance of calling a person a "sensitized responder" is very small (less than one in ten thousand). A person may be a "sensitized responder" and not have CBD. (See Appendix B and C for illustrations)

    9.1. The BeLPT shall be reported as unacceptable if any of the following criteria are **NOT** met.

        9.1.1. Background counts should be within the acceptable level for the counting instrument (determined during the machine calibrations step performed as part of the quality control procedures).

        9.1.2. The control well counts should be at least 2 times higher than the background counts (as a qualitative measure).

        9.1.3. Mitogen-stimulated wells should clearly demonstrate lymphocyte proliferation [standardized Ln(SI) greater than 3.0].

        9.1.4. The internal variability for control wells or beryllium stimulated wells is acceptable. The standard deviation of natural log transformed count data for control wells should be less than 0.95, and for the Be treated wells it should be less than 1.5 when 12 control wells are used and 4 beryllium stimulated wells are used for each treatment group. These values are guidelines that are most useful when there is no evidence for a beryllium response.

        9.1.5. At least half of the standardized Ln(SI)s are greater than -3 (i.e., no strong evidence of cell killing).

    9.2. A BeLPT shall be reported as an abnormal test if both of the following criteria are met. (Appendix C contains three examples of the summary results illustrating a normal test, an abnormal test, and a borderline test that requires further evaluation.)

        9.2.1. The standardized maximum Ln(SI) is greater than 3.l.

        9.2.2. At least two standardized Ln(SI)s are greater than 2.5 indicating a positive response to beryllium.

        9.2.3. A large positive value of the standardized maximum SI indicates a "biological positive" test, i.e., it indicates by how many standard deviations this metric exceeds the typical maximum response for normal individuals in that serum.

Two or more large standardized Ln(SI)s indicate a "statistically positive" test. For the BAL-LPT, only 9.2.2. needs to be satisfied for an abnormal test.

9.3. A BeLPT shall be reported as normal if neither 2.2.1 or 2.2.2 is satisfied.

9.4. If either 2.2.1 or 2.2.2 is satisfied, but not both, then the test shall be reported as borderline. Further interpretation is required based on a more detailed evaluation of the test results, usually in combination with repeat testing (see Appendix B for details).

10. REFERENCES

10.1. Epstein, P.E. et al. Bronchoalveolar lavage of a patient with chronic berylliosis. Ann. Int. Med. 97:213-l 6, 1982.

10.2. Kreiss, K. et al. Screening blood test identifies subclinical beryllium disease. J. Occup. Med. 35:267-74, 1993.

10.3. Kreiss, K., et al. The epidemiology of beryllium sensitization and disease in nuclear workers. AM. Rev. Resp. Dis. 148:985-91, 1993.

10.4. Richeldi L., et al. HLA-DPBl glutamate 69: a genetic marker of beryllium disease. Science 262:242-244, 1993.

10.5. Rossman, M.D., et al. Proliferative response of bronchoalveolar lymphocytes to beryllium. Ann. Int. Med., 108:687-693, 1988.

10.6. Saltini C., et al. Maintenance of alveolitis in patients with chronic beryllium disease by beryllium-specific helper T cells. New Engl. J. Med., 320:1103-l 109, 1989.

APPENDIX A

ABREVIATED LISTING OF REQUIRED EQUIPMENT AND SUPPLIES

Plastic disposable sterile culture tubes, 16 x 125 mm, with screw cap

Pipettes, serological with plug, plastic, sterile and individually wrapped (1, 5, and 10 ml)

Multi-channel micropipettors calibrated to deliver microliter quantities of medium and reagents

Microtest 111 tissue culture plate and lid, 96 well, 0.32 ml/well (round or flat bottom)
Note: Until such time as a definitive study showing a conclusive advantage to round vs. flat, either is usable without prejudice.

Centrifuge

CO_2 incubator

Laminar flow hood

Sterile, cotton plugged Pasteur pipettes

Hemocytometer or Coulter Counter

Microscope

15 ml and 50 ml conical centrifuge tubes

96 well cell harvester

Gas ionization or liquid scintillation counter for detecting beta emissions from tritiated thymidine.

Note: The items above are recommended for simplicity. Equivalents are acceptable. Each laboratory conducts usual procedures to assure satisfactory operation of all equipment.

OTHER NOTES OF INTEREST

MSD sheets are available for all chemicals and laboratory reagents and should be read by all personnel before performing this assay.

RPMI-1640, L-glutamine, penicillin-streptomycin, sterile phosphate buffered saline solution and Ficol-Hypaque are sterile tissue culture reagents. When used with appropriate tissue culture laboratory practices they pose no known safety, health, or disposal hazards.

Beta Plate Stint or its equivalent is a biodegradable scintillation fluid. It is stored and used in a chemical fume hood with appropriate protective clothing, gloves, and face shield. If a direct counting method is employed, this does not apply.

Human serum, type AB has been tested by an FDA approved method and found non-reactive for the presence of HBsAG and antibody to HIV by the supplier. However, it is derived from human source material and will be handled observing the same safety precautions used when handling any potentially infectious material.

Tritiated thymidine emits low energy beta radiation, and shall be used with appropriate protective clothing, gloves, and face shield. Policies for the use and disposal of radioactive reagents and laboratory ware are found in the guidelines prepared by the institution's safety officer and shall comply with accepted practices.

Beryllium sulfate is an extremely hazardous chemical. It is, based upon animal data, assumed to be a potential human carcinogen. It is a strong irritant; contact with skin, eyes, and mucous membranes must be avoided. Consult MSD sheet before handling this chemical.

Computer software written in language Splus is available for performing the calculations described in this specification (see appendix of ORNL-6818 and http://www.epm.ornl.gov/-frome/BeLPT). The procedure, however, is sufficiently straightforward so that simple programming can be used to implement it in any spreadsheet.

Two Excel programs that use the data in Table B-I to illustrate the calculations are available on page 12 of the Tritiated Thymidine Beryllium Lymphocyte Proliferation Test Electronic notebook at URL: http://www.epm.ornl.gov/~frome/BE/tnote.html

APPENDIX B

DETAILS OF THE CALCULATIONS FOR THE LPT

As the result of biological variability in the well counts there are different levels of uncertainty present in each LPT. This internal variability is described by the standard deviation of the Ln well counts, and is equivalent to the coefficient of variation on the original scale. The "internal analysis" of the LPT is based on estimates of the Ln(SI)s and their standard errors. These estimates are calculated using the least absolute values (LAV) method described in detail in Environmental Health Perspectives Supplement, 1996 (EHP96). This approach only requires the ability to calculate medians and can be done in a spreadsheet (e.g., Excel) or statistical program (e.g. Splus). A summary of the assumptions for this analysis and alternative methods that could be used are provided in Section B-8. Results of using these methods on LPT data are provided in reports listed in Section B.10.

The example that follows is a tritiated thymidine BeLPT using the assay design described in EHP96 and is provided to illustrate the general procedure. The only quantity that depends on the assay design (i.e. the number of replications for the control wells and/or the treated wells) is the standard error of the Ln(SI) in step B.5. The same methods can be used for the BAL-LPT. The standardized Ln(SI)s are used to determine if stimulated cells show a positive response. They can also be used in the evaluation of new technicians (see 6.3.4), and in the selection of a new serum (6.3.1).

B.1. Calculate the Ln of the well counts in Table B-I and record in columns 2-5 of Table B-II.

Table B-I
BeLPT Count Data

Treatment Group	**Well Counts**			
Day5 controls	1220	2391	1774	947
Day5 controls	1499	1568	1410	1131
Day5 controls	969	2265	1743	728
Day5 Be1	1777	1890	1702	1885
Day5 Be10	3368	7221	1473	3097
Day5 Be100	3631	3655	2452	1634
Day7 controls	3616	17410	3989	3144
Day7 controls	669	1257	1497	4460
Day7 controls	2897	4174	1366	1152
Day7 Be1	1670	2186	629	1264
Day7 Be10	330	598	254	264
Day7 Be100	3611	4436	14452	14892
PHA	102160	44223	59344	51088
CONA	115673	104146	252237	159421

B.2. For each Treatment Group calculate the median of the Ln counts (see Table B-II column 6).

Table B-II

Treatment Group	Ln (well counts)				Median
Day5 controls	7.1066	7.7795	7.4810	6.8533	7.2819
Day5 controls	7.3126	7.3576	7.2513	7.0309	7.2819
Day5 controls	6.8763	7.7253	7.4634	6.5903	7.2819
Day5 Be1	7.4827	7.5443	7.4396	7.5417	7.5122
Day5 Be10	8.1221	8.8847	7.2951	8.0382	8.0801
Day5 Be100	8.1973	8.2039	7.8047	7.3988	8.0010
Day7 controls	8.1931	9.7648	8.2913	8.0533	8.0123
Day7 controls	6.5058	7.1365	7.3112	8.4029	8.0123
Day7 controls	7.9714	8.3366	7.2196	7.0493	8.0123
Day7 Be1	7.4206	7.6898	6.4441	7.1420	7.2813
Day7 Be10	5.7991	6.3936	5.5373	5.5759	5.6875
Day7 Be100	8.1917	8.3975	9.5786	9.6086	8.9880
PHA	11.534	10.697	10.991	10.841	10.916
CONA	11.658	11.553	12.438	11.979	11.819

B.3 For each beryllium concentration calculate the Ln(SI) by subtracting the median of the control wells from the median of the Be stimulated wells, e.g., for Day5 Be100 the Ln(SI) = 8.0010 - 7.2819 = 0.7191 and the SI is exp(0.7191) = 2.05.

B.4 Calculate the standard deviation of the Ln counts (corresponds to CV on original scale). In EHP96 this is referred to as "phitilde". The median absolute deviation (MAD) estimate given on page 960 (called here S_m--- the MAD estimate of the standard deviation), is

$S_m = 1.48 * \text{median}[\ \text{absolute(residual)}\ ] * \sqrt{[n/(n-p)]}$ where:

absolute(residual) = the absolute value of the (Ln well count - the median Ln well count);
n the number of data values is 24, if 12 control wells and 12 exposed wells; and
p the number of parameters is 4, if 1 median for control wells and 3 medians for exposed wells.

A separate estimate of S_m is calculated for Day 5 and Day 7, since it has been observed that there is generally more variability on Day 7. Using Day 5 as an example, the residuals are listed in Table B-III.

Table B-III

	Residuals for Day 5			
Day5 controls	-0.1753	0.4976	0.1991	-0.4286
Day5 controls	0.0307	0.0757	-0.0306	-0.2510
Day5 controls	-0.4056	0.4434	0.1815	-0.6916
Day5 Be1	-0.0295	0.0321	-0.0726	0.0295
Day5 Be10	0.0420	0.8046	-0.7850	-0.0419
Day5 Be100	0.1963	0.2029	-0.1963	-0.6022

The median absolute value of the residuals is 0.1963, and

$S_m = 1.48 * 0.1963 * \sqrt{(24/20)} = 0.3183,$

B.5 Calculate the standard error of the Ln(SI):

StErr[Ln(SI)] = S_m * sqrt(pi/2) * constant.

For this assay design (12 control wells and 4 beryllium stimulate wells) the constant is 0.577. If, for example, 8 control wells had been used the constant would be 0.612 (see EHP96 page 960 for details).

On Day 5 the standard error [Ln(SI)] = 0.3182531*1.253*0.577=0.230.

B.6 Divide the Ln(SI) by its standard error to obtain the standardized Ln(SI):

standardized Ln(SI) = Ln(SI) / standard error[Ln(SI)] .

For Day 5 Be100 the standardized Ln(SI) = 0.7197/0.230 = 3.13. Dividing each Ln(SI) by its standard error results in a statistic that is in "standard measure", having mean 0 and standard deviation 1 (see B.10.6, p. 631), i.e., a standardized Ln(SI).

B.7 The results of the calculations for each treatment on Day 5 and Day 7 are recorded.

B.8 Assumptions and Alternative Methods of Analysis for the Tritiated Thymidine BeLPT.

B.8.1 The LAV analysis is based on the assumptions that:

a) Ln of the well counts are normally distributed;
b) standard deviations of Ln counts are constant within harvest days;
c) multiple outliers may be present in the Ln well counts; and
d) if "responder cells" are present an increase in cell proliferation relative to the control wells will occur in cultures with beryllium.

B.8.2 If outliers are not considered to be a problem, then conventional least squares (i.e., using means instead of medians in B.2 and the usual estimate of the standard deviation in B.4) could be used. Also, note the sqrt(pi/2) is not included in StErr[Ln(SI)].

B.8.3 An alternative outlier resistant method that calculates estimates of the Ln(SI)s and their standard errors could also be used. An example of another outlier resistant method is given in EHP96 (see Quasi-likelihood estimation). Alternative approaches to robust regression are described in Chapter 25 of “Applied Regression Analysis”, Third Edition (see Reference B.10.1).

B.8.4 The only requirement if another method is used is that appropriate values of the Ln(SI)s (B.3) and their standard errors (B.5) shall be used in B.6 to obtain the values of the standardized Ln(SI) for use in 10.3.2 to determine if two or more beryllium treated wells show a positive response.

B.9 The results of all of the calculations and additional statistics are combined into a single laboratory LAV report (see Table B-IV)

Note that S_m and the residuals in the top panel of Table B-IV have been multiplied by 100, i.e. they are in Ln percent units. The estimates of S_m for the control wells and treated wells for Day 5 and Day 7 with and without "pooling" are provided in the last panel of Table B-IV. An "overall" pooled estimate is also provided. These values are used to calculate the standard error[Ln(SI)] and to evaluate the amount of variation within control and treated groups on Day 5 and 7.

The LAV report is used for quality control and to help in the interpretation of LPTs that are not confirmed abnormals or normals. The LPT in Table B-IV has two large standardized Ln(SI) s (D5be10 and D5be100) indicating a positive test (see Sec 11.2 b).The standardized maximum SI for this test, (see Sec 10.9) is:

standardized maximum SI = (0.98 - M)/ SD = (0.98 - 0.081)/0.34 = 2.64.

This is below the cut point of 3.1 (see Sec 13.1) for the reference data set. The values of S_m are high on Day 7 and the standardized Ln(SI) for D7be10 is -3.98, indicating cell killing in at least one well. This test was interpreted as "borderline" by the laboratory, and subsequent follow-up found this patient to be SENSITIZED.

Table B-IV
LPT Analysis Using Least Absolute Values -- EHP 1996

Treatment Group	Well Counts				Fitted Value	S_m	Residuals			
Day5 controls	1220	2391	1774	947	1453.8	34.9	-18	50	20	-43
Day5 controls	1499	1568	1410	1131	1453.8	34.9	3	8	-3	-25
Day5 controls	969	2265	1743	728	1453.8	34.9	-41	44	18	-69
Day5 Be1	1777	1890	1702	1885	1830.2	5.3	-3	3	-7	3
Day5 Be10	3368	7221	1473	3097	3229.7	70.8	4	80	-79	-4
Day5 Be100	3631	3655	2452	1634	2983.8	34.2	20	20	-20	-60
Day7 controls	3616	17410	3989	3144	3018.0	84.5	18	175	28	4
Day7 controls	669	1257	1497	4460	3018.0	84.5	-151	-88	-70	39
Day7 controls	2897	4174	1366	1152	3018.0	84.5	-4	32	-79	-96
Day7 Be1	1670	2186	629	1264	1452.9	46.9	14	41	-84	-14
Day7 Be10	330	598	254	264	295.2	22.4	11	71	-15	-11
Day7 Be100	3611	4436	14452	14892	8006.8	103.7	-80	-59	59	62
PHA	102156	44221	59346	51090	55063.5	25.2	62	-22	7	-7
CONA	115673	104146	252237	159421	135796.6	36.4	-16	-27	62	16

Fitted Value = exp[median(Ln z)] for each treatment group
NOTE: S_m and residuals are in Ln % units

STIMULATION INDICES (SI)

	Day 5			Day 7				
	D5Be1	D5Be10	D5Be100	D7Be1	D7Be10	D7Be100	PHA	CONA
SI	1.26	2.22	2.05	0.48	0.10	2.65	37.89	93.41
Ln(SI)	0.23	0.80	0.72	-0.73	-2.32	0.98	3.63	4.54
Stan. Ln(SI)	1.00	3.48	3.13	-1.25	-3.98	1.67	15.83	19.76

NOTE: Standardized Ln(SI) = Ln(SI)/Standard Error is Standardized Ln (SI)
Large POSITIVE value (GT 2.5) Indicates POSITIVE Response
Large NEGATIVE value (LT -2.5) Indicates Cell Killing

Summary Statistics S_m (Coefficient of Variation)* for PM1271

Overall:	0.385				
Day 5 Control:	0.349	Day 5 Treated:	0.23	Day 5 Pooled:	0.319
Day 7 Control:	0.845	Day 7 Treated	0.855	Day 7 Pooled:	0.811

*S_m is MAD estimate of the standard deviation on Ln scale (corresponds to CV on original scale)

B.10. REFERENCE DATA SET FOR EACH SERUM

B.10.1. For the tritiated thymidine BeLPT, the laboratory shall maintain a reference data set based on at least 30 acceptable (Section 9.8) unexposed and/or normal BeLPTs for each serum. The data for each BeLPT in the reference data set shall be retained and used to evaluate all subsequent BeLPTs in that serum. The maximum SI for these "normal" individuals is assumed to follow a normal distribution on the Ln scale, i.e. maximum Ln(SI) follows a normal distribution with a median and standard distribution. Estimates of the median and standard deviation are calculated as follows:

B.10.2. Calculate the Ln of the maximum SI for each BeLPT in the reference data set;

B.10.3. Calculate M the median of the Ln (maximum SI) values;

B.10.4. Calculate SD the outlier resistant (i.e MAD) estimate of the standard deviation for the Ln(maximum SI) values;

B.10.5. The values of M and SD for each lot of serum are used to calculate the standardized maximum Ln(SI) for each patient tested in that serum. The standardized maximum Ln(SI) is obtained by subtracting the value of the median of reference data set from the maximum Ln(SI) and then dividing by the standard deviation from the reference data set.

B.10.1 References

B.10.1 Draper, N.R. and Smith, H., (1998), Applied Regression Analysis. Third Edition, NY, J. Wiley & Sons.

B.10.2 Frome, E., Cragle, D., Littlefield, L.G., and Colyer, S. (2000), Results of the Analysis of the Blood Lymphocyte Proliferation Test Data From the Oak Ridge Y-12 Study, DRAFT ORNL Report (under revision).

B.10.3 Frome, E.L., Smith, M., Littlefield, G., Neubert, R., and Colyer, S. (1996) Statistical Methods for the Blood Beryllium Lymphocyte Proliferation Test, Environmental Health Perspectives, Vol 105, Supplement 5, 957-968.

B.10.4 Frome, E.L., L. S. Newman, and M. M. Mroz (1997) Results of the Analysis of the Blood Lymphocyte Proliferation Test Data From the National Jewish Center, ORNL/TM-13338, pp 1-36.

B.10.5 Frome, E.L., Smith, M., Littlefield, G., Neubert, R., and Colyer, S. (1994) Statistical Methods for the Analysis of a Screening Test for Chronic Beryllium Disease, ORNL-6818), pp 1-61.

B.10.6 Kotz, S. and Johnson, N.L. (1988) Encyclopedia of Statistical Science, Vol 8, John Wiley & Sons, New York.

References B.10.2-5 are available at www.epm.ornl.gov/~frome/BeLPT/

APPENDIX C

The following three tables contain summary results for three BeLPTs from the Oak Ridge Y-12 Study (see B.10.2.). The tables illustrate summary information that could be provided in a report to the referring physician for each BeLPT. The criteria in Results Section 9 were applied to each of the tests. Table B-I illustrates results for a normal BeLPT. The value of the standardized maximum SI was 0.67 and all standardized Ln(SI)s in column 6 were below 2.5. The standardized Ln(SI) for the highest concentration of Be sulfate on harvest Day 7 shows an indication of cell killing (standardized Ln(SI) = -3.57). The internal variability on Day 7 is on the high side for Be-stimulated wells which is also an indication of cell killing.

Table C-I-- Normal BeLPT

Name:NA **LabID PMC073** **Date:**

	Reps	**Median**	**SI**	**S_m**	**Standardized Ln(SI)**
Control	**12**	**2152**	**NA**	**0.30**	**NA**
D5Be1	4	2935	1.36	0.25	1.58
D5Be10	4	1780	0.83	0.31	-0.97
D5Be100	4	1670	0.78	0.10	-1.29
Control	**12**	**1366**	**NA**	**0.40**	**NA**
D7Be1	4	1381	1.01	1.09	0.03
D7Be10	4	1190	0.87	0.63	-0.38
D7Be100	4	371	0.27	1.04	-3.57
PHA	4	31556	43.98	0.16	19.26
CONA	4	76344	106.40	0.16	23.76

The standardized maximum Ln(SI) for this patient is 0.67.

INTERREPTATION: This patient has a normal response to beryllium.

Table C-II illustrates an abnormal BeLPT. The value of Standardized maximum Ln(SI) is 7.8 and all six standardized Ln(SI) s in Column 6 are greater than 2.5

Table C-II-- Abnormal BeLPT

Name:NA **LabID PM1296** **Date:**

	Reps	**Median**	**SI**	**S_m**	**Standardized Ln(SI)**
Control	**12**	**1296**	**NA**	**0.53**	**NA**
D5Be1	4	4083	3.15	0.06	2.93
D5Be10	4	6415	4.95	0.67	4.09
D5Be100	4	19918	15.37	0.38	6.99
Control	**12**	**748**	**NA**	**0.51**	**NA**
D7Be1	4	3586	4.80	0.63	3.61
D7Be10	4	6340	8.48	0.47	4.92
D7Be100	4	8914	11.92	0.79	5.70
PHA	4	169781	393.02	0.25	15.27
CONA	4	180308	417.39	0.13	15.43

The standardized maximum Ln(SI) for this patient is 7.80.

INTERREPTATION: This patient has an abnormal response to beryllium.

Table C-III contains the summary results for the detailed example in Appendix B. The value of the standardized maximum Ln(SI) = 2.63, below the cut point 3.1 (see 10.3.1.). There are two standardized Ln(SI) s greater than 2.5 on Day 5 (see column 6) which indicates a positive response. Day 7 shows high internal variability in both the control well (S_m = 0.845) and the Be-stimulated wells (S_m = 0.855) -- See Table B-IV. Also, there is strong evidence for cell killing at Be10 (standardized Ln(SI) = 3.98).

Table C-III-- LPT That Requires Further Interpretation

Name:NA **LabID PM1271** **Date:**

	Reps	**Median**	**SI**	**S_m**	**Standardized Ln(SI)**
Control	**12**	**1454**	**NA**	**0.35**	**NA**
D5Be1	4	1830	1.26	0.05	1.00
D5Be10	4	3230	2.22	0.71	3.48
D5Be100	4	2984	2.05	0.34	3.13
Control	**12**	**3018**	**NA**	**0.85**	**NA**
D7Be1	4	1453	0.48	0.47	-1.25
D7Be10	4	295	0.10	0.22	-3.98
D7Be100	4	8007	2.65	1.04	1.67
PHA	4	55063	113.63	0.25	20.62
CONA	4	135797	280.22	0.36	24.55

The standardized maximum Ln(SI) for this patient is 2.63.

INTERREPTATION: This patient has a borderline response to beryllium on Day 5. Day 7 data is unacceptable due to high internal variability and cell killing. Test should be repeated.